Epitope mapping of the *Mycobacterium bovis* secretory protein MPB70 using overlapping peptide analysis

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The gene encoding the major *Mycobacterium bovis* secreted protein MPB70 was cloned and isolated from a DNA library in λ EMBL 3, and the restriction map of the area of chromosome containing the gene ascertained. After sub-cloning, the complete DNA sequence and predicted amino acid sequence were determined, and from this information a series of overlapping octapeptides encoding all possible linear epitopes of eight or less amino acids were synthesized. These peptides were probed with monoclonal antibodies specific for *M. bovis* and with sera from *M. bovis*-infected cattle. Epitopes defined by this technique were then examined using a substitution analysis that allowed the significance of each amino acid in the putative epitope to be quantified, and the exact specificity of the antibody response for the epitope determined.

Introduction

In many countries, efforts to control or eradicate bovine tuberculosis have been frustrated by the lack of an unequivocal and objective diagnostic test for the disease. Current diagnosis of bovine tuberculosis is by the caudal fold skin test for delayed-type hypersensitivity (DTH), a test analogous to the human mantoux test. In cattle, a DTH reaction to a subcutaneous injection of *Mycobacterium bovis* purified protein derivative (PPD) is indicative of infection with *M. bovis*. The PPD produces a swelling at the site of injection, normally the caudal fold, in infected animals. Monitoring of the swelling is done on a subjective basis by the examining veterinarian. The test is neither highly sensitive nor specific, and ideally a simple serological test, such as that used for brucellosis, would be a preferred method of diagnosis.

Serological diagnosis of all mycobacterial diseases is complicated by the large degree of cross-reactivity of many mycobacterial antigens, with both non-pathogenic mycobacteria and bacteria of other genera (Stanford & Grange, 1974; Daniel & Janicki, 1978; Bloom, 1986). The problem is compounded by the spectrum of immune response to mycobacterial infection, where different species and individuals vary in immune reaction to mycobacterial challenge. Some develop high antibody titres with low T-cell reactions, whereas in others the situation is reversed (Thorns & Morris, 1983; Bloom, 1986; Lagrange & Hurtrel, 1988).

Despite the cross-reactivity of mycobacterial antigens, monoclonal antibodies (mAbs) specific to the *Mycobacterium tuberculosis*/*M. bovis* group have been raised and used in competitive assays for *M. tuberculosis*-specific antibody with some success (Hewitt et al., 1982; Ivanyi et al., 1983; Hoeppner et al., 1987). Protein antigens from both *M. leprae* and *M. tuberculosis* have been isolated using species-specific mAbs (Young et al., 1985a, b), but virtually all appear to possess cross-reactive epitopes that would limit their potential as diagnostic antigens (Kadival et al., 1987; Kingston et al., 1987). Analysis of specific epitopes would permit the synthesis of peptides specific to individual mycobacterial species.

Our efforts have been directed towards isolating specific antigens from *M. bovis*, the causative agent of bovine tuberculosis, to improve the diagnosis of this disease. We have isolated and cloned the antigen MPB70 (Radford et al., 1988; Fifis et al., 1989), which is
considered to be largely a specific antigen of *M. bovis*, although some cross-reactivity with *Nocardia* has been reported (Harboe & Nagai, 1984; Harboe et al., 1986). MPB70 was first isolated from *M. bovis* BCG (Nagai et al., 1981), and is a major antigen produced by some BCG strains (Miura et al., 1983; Abou-Zeid et al., 1986). MPB70 is secreted from *M. bovis* strains, as evidenced by its predominance in culture filtrate preparations (Harboe & Nagai, 1984; Abou-Zeid et al., 1986). It is around 18–23 kDa as estimated by denaturing polyacrylamide gel electrophoresis, although it appears to be anomalous in its gel mobility (Nagai et al., 1981; Haslov et al., 1987; Abou-Zeid et al., 1987; Fifis et al., 1989). MPB70 antigen is a prominent antigen in PPD preparations of *M. bovis*. Monoclonal antibody analysis has shown that it contains at least three separate *M. bovis*-specific epitopes (Wood et al., 1988). Cattle infected with *M. bovis* often have serological responses to MPB70, and it is a powerful T-cell stimulator in these animals (Fifis et al., 1989). A large number of *M. bovis* strains isolated from tuberculous cattle have been shown to produce the MPB70 antigen (Corner et al., 1988). Definition of the specific epitopes of MPB70 is a step towards accurate diagnosis of bovine tuberculosis, enabling these epitopes to be synthesized and used as antigens in serological diagnostic assays. They might also play a role in human tuberculosis diagnosis by distinguishing active tuberculosis from a BCG vaccine response.

**Methods**

*Bacteria and cloning vectors*. *M. bovis* AN5 was from the Commonwealth Serum Laboratories, Parkville, Australia, originally obtained from the Ministry of Agriculture Weybridge Laboratories, UK. *Escherichia coli* strains Y1090, Y1089, NM538 and NM539 have been previously described (Young & Davis, 1983; Frischaufl et al., 1983). λ phages gt11 and EMBL3 were from Promega Biotec (Wisconsin, USA), and M13 mp18 was obtained from Bresatec (South Australia). The origin and sequence of λ gt11 clone C4a has been previously published (Radford et al., 1988).

**DNA techniques**. All enzymes and phage vectors were purchased from Promega Biotec except where otherwise noted. *M. bovis* DNA extraction was done using the method of Shoemaker et al. (1986). Cloning and DNA techniques were as described by Maniatis et al. (1982). Sequence analysis was by the primer extension dideoxy termination method of Sanger et al. (1980), after DNA was cloned in M13 mp18. Both reverse transcriptase and T7 DNA polymerase (Sequenase, USB) were used, and sequencing reactions were analysed on 6% polyacrylamide/8 M-urea gels.

**Antibodies**. The SB series of monoclonal antibodies (mAbs) have been previously described (Wood et al., 1988), and a generous gift from Agen Australia Ltd. Cows named Parade 15, 51 and 270 were naturally infected with *M. bovis* and had previously been shown by serological testing to have high titre against the purified MPB70 antigen (Fifis et al., 1989). Control negative cattle sera were from skin test negative cattle purchased from a tuberculosis-free herd. Peroxidase conjugates against mouse and bovine immunoglobulin were from Kirkegaard and Perry Laboratories (Maryland, USA) and were used at optimum concentration.

**Synthesis of overlapping peptides**. Peptides were synthesized on polyethylene pins as previously described in detail (Geysen et al., 1987). The t-butylxycarboxyl (BOC) deprotection cycle was modified to include the use of methanol rather than dichloromethane as the solvent for the neutralization and washing steps. These changes had no detectable effect on the efficiency of peptide synthesis when monitored by amino acid analysis, and were introduced to shorten the BOC-deprotection cycle and make it more economical.

**Enzyme-linked immunosorbent assay (ELISA)**. ELISA tests were carried out as previously described (Geysen et al., 1987) except that colour development was stopped before the completion of the usual 45 min incubation time if the absorbance of the most highly coloured enzyme substrate solution reached approximately 2.0. This precaution was taken in order to reduce the possibility of non-linearity of the relationship between the amount of enzyme conjugate bound and ELISA absorbance. For the network analysis, ELISA results were regarded as significant when the absorbance was three standard deviations above the mean of the values of the lowest 25% of signals. The epitope was defined as the group of peptides with the highest significant mean signals; in every case the mean values of the group of peptides defining the epitopes were greater than three standard deviations higher than the mean absorbance of all the peptides.

**Determination of amino acids essential to antibody binding**. The contribution of individual residues to the binding properties of an epitope was examined using the replacement net approach (Geysen et al., 1984, 1987). Briefly, a set of analogues of a known epitope were synthesized, in which each residue was replaced with all 19 alternative genetically coded amino acids, one at a time. Thus, for a hexapeptide, a set of 114 singly-substituted analogues was synthesized and tested by ELISA, along with six replicates of the original or 'parent' peptide. The amounts of antibody bound by each of the analogues then comprise a replaceability pattern for the residues in that epitope.

**Results and Discussion**

*M. bovis* AN5 DNA was partially digested with restriction enzyme Sau3A and size-selected on an agarose gel for fragments greater than 10 kbp, which were then extracted from the gel using Geneclean (Bio101, California, USA). Inserts were ligated with λ EMBL3 DNA that had been prepared with BamHI cohesive ends. Packaging and plating of the phage gave 1·5 × 10⁶ p.f.u. and plating the phage on the recombinant restrictive host, *E. coli* NM539, revealed that the library was greater than 50% recombinant phage.

The long fragment library of *M. bovis* was examined using the MPB70 partial clone C4a as a hybridization probe (Radford et al., 1988) and two phages reactive with this clone were isolated. The restriction map of the MPB70 region of the *M. bovis* chromosome was then established using these two clones; it is shown in Fig. 1. Southern blot analysis of *PstI*-digested AN5 DNA had shown that the C4a clone hybridized with a 1·85 kbp *PstI* fragment, which was found to be present in both reactive phages. This fragment was subcloned in M13 mp18 and
DNA was sequenced using primers established from the previously sequenced C4a clone.

The sequence was confirmed by subcloningMspI fragments of the gene and sequencing the complementary strand. The sequence coding for the mature MPB70 protein is given in Fig. 2. When synthesized, MPB70 has the 30 amino acid secretory signal sequence on its amino terminus as shown in Fig. 2. The signal sequence is cleaved off in the mature protein and is not relevant to the antibody response.

The total size of mature MPB70 protein predicted from the DNA sequence is 16 kDa, smaller than the apparent 18–23 kDa size of natural MPB70 (Harboe & Nagai, 1984; Abou-Zeid et al., 1987, Fifis et al., 1989) as assessed by gel electrophoresis; this suggests either that MPB70 from M. bovis is post-translationally modified prior to secretion or that it has aberrant mobility in denaturing polyacrylamide gel electrophoresis. Deviation from the expected size is not uncommon in M. bovis antigens; the mba gene codes for a protein around 7 kDa smaller than the apparent mobility of the natural antigen (Thole et al., 1988), and the gene for the MPB64 antigen codes for a protein of 22.4 kDa (Yamaguchi et al., 1989), compared with the 27 kDa suggested by Abou-Zeid et al. (1987) from SDS gel studies.

The amino acid sequence inferred corresponds roughly up to amino acid 115 on Fig. 2 with the two different protein sequences of MPB70 published by Patarroyo et al. (1986a, b) determined by amino acid sequencing of MPB70 derived from M. bovis BCG. From amino acid 115 to 163 there is very little to no correspondence with the data of Patarroyo et al. (1986a, b).

Codon usage in the MPB70 gene reflects the high G + C content (66 mol%) of M. bovis (Imaeda et al., 1982) with a preference for G or C in the third position. In this it is similar to the M. bovis mba 65 kDa antigen, the alpha antigen and the MPB64 genes (Thole et al., 1988; Yamaguchi et al., 1989), although codon usage was not identical between any two of these genes. No biological function has been ascribed to the MPB70 protein, and its level of production has not been linked to any M. bovis characteristic. A computer search of a protein database (NBRF-PIR R14.0, LKB, Sweden) failed to show sufficient similarity for any inferences of protein function to be drawn.

Epitope mapping of M. bovis MPB70 protein

To enable the exact definition of the monoclonal and polyclonal epitopes of MPB70, a duplicate series of overlapping octapeptides were synthesized to mimic all possible linear epitopes of MPB70 based on the predicted protein sequence. These peptides were bound to polyethylene pins arranged to fit the wells of a standard microtitre tray. This allowed both simple synthesis and ELISA analysis to be performed to establish which peptide sequence was recognized by the M. bovis-specific mAbs and by sera from M. bovis-infected animals.

Previous antibody competition studies have shown that the SB series of mAbs recognize at least three distinct epitopes (Wood et al., 1988). Two mAbs recognizing each epitope were chosen for analysis on the MPB70 octapeptide net. SB2 and SB3 were in group 1, SB6 and SB8 in group 2, and SB9 and SB10 in group 3. ELISA quantification of the binding of the SB mAbs to the peptide network showed that only one of the three different epitopes established previously was present. As all the SB mAbs recognize recombinant MPB70, this would exclude the possibility that they recognize possible non-protein moieties and suggests that the remaining two epitopes for the mAbs SB2/SB3 and SB6/SB8 are conformational and dependent upon secondary structure. Alternatively, the antibodies may require a peptide longer than the octapeptides used for binding. The epitope size is a critical factor in this study since there is the possibility that there are amino acids that contribute to, or inhibit, antibody binding outside the ‘core’ sequence that is present on the octapeptide.

The results of the network analysis for SB9 and SB10 are shown in Fig. 3. SB9 showed strong recognition of octapeptides 15–19, indicating that the epitope is located between amino acids 15 and 22 of MPB70. The peptides starting at positions 18 and 19 gave lower signals than the three preceding, suggesting that the latter peptides do not contain the full epitope. This implies that the threonine at position 17 forms part of the epitope, but asparagine (15) and proline (16) are less critical, and the epitope is contained in the sequence NPTGPASV.

mAb SB10 gave a very definite signal on octapeptides 23–27, suggestive of an epitope contained between amino acids 23 and 30 within the sequence QGMSQDPV. As the signal was of similar magnitude in all reactive peptides in the 23–27 group, and dropped to background levels on peptide 28, it can be inferred that the epitope is present on all octapeptides from 23 to 27. The overlap between peptides 23 and 27 indicates that the critical epitope is QDPV. Some other octapeptides showed single spikes of reactivity; these were not considered in terms of defining the epitope as they were not flanked by similarly reactive octapeptides. Each epitope differs from its neighbour only by the
addition of one amino acid to the carboxyl terminus and deletion of one at the amino terminus, and thus true epitopes are indicated by groups of reactive peptides.

All mAbs tested on the network showed minor peaks of binding between octapeptides 110–116 and 146–153. These signals were mostly below minimum significance levels and were considered to be reflective of adhesion of the mAbs due to factors other than antibody–epitope binding.

The network data illustrate that although the mAbs SB9 and SB10 exclude each other in competition studies (Wood et al., 1988), they actually recognize separate epitopes on the MPB70 molecule, these being separated by four amino acids. Competition between the two mAbs must be based on steric properties rather than direct competition for the same binding site, and this is reflected by the observation that the mAbs can be distinguished by their recognition of different λ gt1 clones of MPB70 (Radford et al., 1988).

Identification of epitopes recognized by cattle sera

The reactivity of serum from an infected cow with the MPB70 octapeptide network is shown in Fig. 4. Animal Parade 51 has a high titre to native MPB70 antigen, and reacted strongly with octapeptides 32–36, indicating a dominant epitope between amino acids 32 and 39 within the sequence VAASNNPE, with the critical sequence being SNNPE. Smaller reactions occurred with octapeptides 21–25, 111–117 and 73–77. The latter group was being SNNPE. Smaller reactions occurred with octapeptides 32–35 and Parade 270 with reactions of sera from two other infected cattle with the network between peptides 20 and 60. Both of these animals showed reactions similar to the Parade 51 animal, animal Parade 15 reacting with peptides 32–35 and Parade 270 with...
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Fig. 3. Scans of octapeptides of the MPB70 protein with SB mAbs. Peptides were reacted with a 1/2000 dilution of the SB series mAb (ascites) and antibody binding ascertained by ELISA (Geysen et al., 1987). Each peptide is identified by both the number in the mature MPB70 sequence and the single-letter code of its amino-terminal residue. The signal is measured in ELISA absorbance units.

Fig. 4. Scans of octapeptides of the MPB70 protein with sera from *M. bovis*-infected cattle. Peptides were reacted with a 1/1000 dilution of the serum and antibody binding ascertained by ELISA (Geysen et al., 1987). Each peptide is identified by both the number in the mature MPB70 sequence and the single-letter code of its amino-terminal residue. The signal is measured in ELISA absorbance units. A full scan is shown for the Parade 51 serum, and the scan from amino acid 20 to 68 for Parade 15 and 270. Neither of these last two sera showed significant reactions with any of the octapeptides outside this range.
peptides 32–36. Although these animals gave lower signals than Parade 51, for both animals the mean absorbance of the epitope groups was more than four standard deviations above the mean absorbance of all the peptides for that sera. No other comparable peaks of reaction were seen using these sera on the total network.

Definition of the mAb epitopes by the replacement net method

Epitopes were analysed for the significance of each amino acid to the antibody-binding reaction by replacement net assay (Geysen et al., 1987). In this system an array of peptides were synthesized where each amino acid in the epitope was substituted by all other possible amino acids, while the rest of the amino acids comprising the putative epitope remained constant. All possible variations were then tested in an ELISA assay for the binding of antibody. In this manner the relative significance of each amino acid to the epitope was ascertained, and amino acids critical for recognition determined (Fig. 5).

It can be seen that there was an almost absolute dependence on amino acids 18 (G), and 21 (S) for the binding of SB9, with the valine at position 22 also being significant but capable of substitution by some other uncharged amino acids. Amino acids 16, 17, 19 and 20 were less critical to the binding of the mAb, although there were some substitutions that destroyed recognition. Thus the SB9 epitope can be seen as XXGXXSV, where X represents virtually any other amino acid. The binding of SB9 to peptide 17 (sequence TGPASVQG) in the network analysis shows that the epitope only requires one amino acid before the critical glycine and can thus be reduced to XGXXSV. This may explain the single spike of binding at octapeptide 142 (Fig. 3), which is explicable when the replacement data are examined. Octapeptide 142 is CGGVSTAN; written XGXXSTXX this is a close match to the defined epitope XGXXSV. As the last valine can be substituted for threonine without signifi-
cantly affecting recognition (Fig. 5) then the SB9 epitope has possibly been mimicked in peptide 142.

The same analysis of the SB10 mAb revealed absolute dependence on amino acids 27 (Q) and 29 (P), with slightly less dependence on 28 (D) and 30 (V). Possible replacements in the latter two did not appear to fall exclusively in any group of amino acids, suggesting that the amino acids at this position do not contribute to antibody binding directly but impose steric or conformational restraints on the epitope. Amino acids 26 (S), 31 (A) and 32 (V) appear totally unrelated to antibody binding. This leaves an epitope of QDPV for the SB10 mAb as suggested by the network analysis.

**Definition of the polyclonal epitope by the replacement net method**

It might be expected that the polyclonal sera would display more complexity than the mAbs in their replacement networks, and that there would be less dependence on particular amino acids for binding of antibody, but this was not the case. Fig. 5 shows the absolute requirement of the polyclonal response of animal Parade 51 for amino acids 36 (N) and 38 (P), with stringent requirements existing at amino acids 37 (N) and 39 (E). Asparagine at position 37 had only one real substitute in aspartic acid; at position 39 glutamic acid could be substituted by aspartic acid, suggesting the necessity of an acidic group at this point. Amino acid 35 (S) had many substitutes and 40 (L) was not critical, defining an epitope of XNNPE.

Thus the polyclonal sera showed similar properties to the mAbs in their epitope recognition. Both find some amino acids essential for binding, and lose almost all signal if these are not present. This requirement suggests that most, if not all, antibodies that recognize this epitope stem from the proliferation of a single cell within the animal. If this is not the case then the antibodies recognizing the epitope deriving from a polyclonal set of cells display remarkable similarity in having the same absolute requirements for binding at amino acids 36 and 38.

Probably the most encouraging aspect of the polyclonal response in the cattle analysed is the predominance of one major epitope on MPB70, close to those regions of the protein known to be recognized by *M. bovis*-specific mAbs. If this predominance is also found in other cattle, and the epitope is shown to be specific, then it is possible that this epitope will form the basis of a serological test for bovine tuberculosis. However, given the variable and often poor serological responses of infected cattle (Plackett et al., 1989) it is unlikely that this epitope alone will be adequate as an antigen in ELISA assays. Other MPB70 epitopes could be used; it remains to be seen whether the known specific epitopes recognized by the SB9 and SB10 mAbs will be recognized by sera from infected cattle. Many other antigens of the *M. tuberculosis*/*M. bovis* complex have been isolated from gene libraries using specific mAbs (Young et al., 1985a; Andersen et al., 1988). Epitope mapping, either by the method described in this paper, or by overlapping clone analysis, may identify species-specific epitopes within cross-reactive antigens suitable for inclusion in a serological test for bovine tuberculosis.

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**References**


